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IMMUNE RESPONSE TO POLYPEPTIDYL PROTEINS IN RABBITS TOLERANT TO THE PROTEIN CARRIERS

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INTRODUCTION

THE specificity of immunological tolerance has been considered to reflect the mechanism of recognition between 'self' and 'not-self' antigenic determinants. It is usually postulated that the experimentally acquired immunological tolerance and natural tolerance are based on the same, yet unknown, mechanism.[1] Accordingly, the immunological manifestations of these two systems of tolerance were expected to be basically similar, and an experimental alteration of the tolerant state achieved in one system might be relevant to the other. The present study was carried out with a twofold aim: (1) to study some molecular parameters associated with the breakdown of acquired immunological tolerance; (2) to analyse further the similarity of immunological manifestations of the two systems of tolerance.

Hapten protein conjugates have been previously employed to investigate these two aspects. The induction of tolerance to serum proteins, followed by immunization with the azo-protein conjugates, has been used in studies both of the discrimination between the tolerogenic protein and the hapten component[2–6] and the subsequent effects of such conjugates in terminating the tolerant state.[6,7] Although breakdown of tolerance, usually at low levels, has been reported in a number of investigations,[6–8] the study of the molecular properties of the immunogenic components which determine the termination of tolerance, has, so far, been limited.[7]

Immunological studies of polypeptidyl proteins have shown that the attachment of polypeptide chains to potent protein antigens may change the serological specificity towards the peptide component,[9,10] while their attachment to such poor antigens as gelatin may in some cases (e.g. peptides of tyrosine, tryptophan, phenylalanine,[11] methionine[12]) result in a strong enhancement of immunogenicity.

In the present investigation we tested the immune response to serum proteins modified by attachment of peptide chains, in animals which have either acquired tolerance to the protein carrier or were naturally tolerant to the protein. Rabbits made tolerant to human serum albumin (HSA) by neonatal injections of the antigen were immunized with poly-L-tyrosyl HSA or poly-DL-alanyl HSA. Normal rabbits were similarly treated with poly-L-tyrosyl and poly-DL-alanyl rabbit serum albumins (RSA). The efficacy of polypeptidyl proteins to terminate the acquired unresponsiveness was evaluated with respect to the degree of enrichment of the protein with tyrosine peptides and the chemical nature of the polypeptidyl component. Antibodies formed against the peptide moiety in both tolerant systems were com-

TABLE 1. SYNTHESIS AND CHARACTERIZATION OF POLYPEPTIDYL PROTEINS

A No. and designation of protein derivative enriched with Tyr or Ala	B N-Carboxy-anhydride of Tyr or Ala (g / g protein)	C Percentage of Tyr or Ala in the original protein	D Percentage of Tyr or Ala in the protein derivative	E Enrichment* $\frac{D-C}{100-D}\cdot 100$	F Moles of amino acid attached per mole of protein	G No. of moles of polypeptide per mole of protein	H Average no. of Tyr or Ala residues per peptide chain attached (F/G)
518,pTyrHSA	0·08	4·3	7·0	2·9	12†		
519,pTyrHSA	0·21	4·3	10·5	6·9	28†		
520,pTyrHSA	0·52	4·3	15·3	13·0	53†	32	1·65
525,pAlaHSA	1·0	6·6	24·6	23·9	225†	29	8
523,pTyrRSA	0·31	4·1	16·0	14·2	58†		
539,pAlaRSA	1·5	5·5	30·0	35·0	330†		
514,pTyrGel	0·5	0·42‡	13·4	15·0	92§		
522,pAlaRNase	3·0	6·12	53·0	100·0	177		

* Calculated assuming the original protein as 100 per cent.
† Assuming a molecular weight of 67,000.
‡ From Sela and Arnon.[11]
§ Calculated for 100,000 g.

pared in order to examine the possible similarities in the recognition mechanisms of natural and induced tolerance.

MATERIALS

Human serum albumin (Fraction V, powder) was obtained from Plasma Fractionation Institute of Israel, Jaffa-Tel Aviv. Rabbit serum albumin (Fraction V, powder) and crystallized human serum albumin were purchased from Mann Research Laboratories, New York, N.Y. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Company, Eastbourne, England. Gelatin, U.S.P. granular, was obtained from Fisher Scientific Co., Pittsburgh, Pa. Ribonuclease A 5 × recrystallized, (lot 53 B-1010) was purchased from Sigma, St Louis, Mo.

Polypeptidyl proteins. The polypeptidyl proteins used in this study are listed in Table 1. They were prepared by the reaction of the protein with the appropriate N-carboxy-α-amino acid anhydride[11,13] (N-carboxy-DL-alanine anhydride[14] or N-carboxy-L-tyrosine anhydride[15]). The protein (1 g) was dissolved, in every case, in 0·05 M phosphate buffer pH 7·0 (80 ml); the N-carboxy-α-amino-acid anhydride dissolved in dioxane (40 ml) was added at 5°, and the reaction mixture was kept at 5° for 24 hr, after which it was dialysed against several changes of distilled water. The contents of the dialysis bag were brought to pH 7·0 with a few drops of 1 N sodium hydroxide, centrifuged at 8000 rev/min to remove any small amount of precipitate, and the clear solution was lyophilized. All the materials prepared were obtained in a water-soluble form.

The extent of enrichment of the protein derivatives with the amino-acid residues attached was calculated from the amino-acid content before and after polypeptidylation. For the two protein derivatives 520,pTyrHSA and 525,pAlaHSA, the number of peptide chains attached, on the average, to one protein molecule was obtained by the determination method used for polyalanyl ribonuclease.[16] In this method the lysine content was determined in the polypeptidyl protein before and after deamination with nitrous acid.

Physicochemical characterization. The polypeptidyl protein preparations investigated were subjected to sedimentation and diffusion in the ultracentrifuge. It was predicted from a theoretical analysis of the kinetics of polymerization of linear and multichain polyamino acids that both linear and multichain polymers of amino acids should possess a relatively narrow distribution of molecular weight.[17,18] This was demonstrated through physicochemical studies of polymers[19-21] as well as chromatographic and ultracentrifugal studies of polyalanyl ribonuclease.[16]

Sedimentation experiments were performed on 0·1 per cent–1·0 per cent solutions of the protein derivatives in 0·1 M phosphate buffer pH 7·0, while the diffusion experiments were carried out on 0·3–1·0 per cent solutions in the same buffer. The extrapolated sedimentation and diffusion coefficients are given in Table 2. A partial specific volume of 0·73 was used in the calculation of the molecular weights (the partial specific volume of human serum albumin is 0·736,[22] of tyrosine residue, 0·71[23] and of alanine residue, 0·72).[19] The molecular weights calculated from the physicochemical measurements are listed in Table 2, and compared with molecular weights calculated from chemical analysis. Data for human serum albumin are given for the purposes of comparison.

METHODS

Induction of tolerance. Every litter of newborn rabbits us[illegible]ed equally into an experimental group and a control group. The expe[illegible]imals were injected intraperitoneally with 40 mg of antigen within 24 hr after birth, another 40 mg a week later, and a third dose of 60 mg at the age of 60 days.

Immunization procedure. The antigens used were incorporated in a water-in-oil adjuvant mixture.[23] Equal parts of 3·0 per cent antigen solution in aqueous 0·9 per cent sodium chloride and ready-prepared complete Freund adjuvant from Difco Laboratories, Detroit, Mich., were homogenized by repeated filling and forcible ejection from a syringe.

TABLE 2. PHYSICOCHEMICAL PROPERTIES OF SOME PEPTIDYL PROTEINS

No. and designation of protein derivative enriched with Tyr or Ala	10^{13} Sedimentation coefficient ($S_{20,w}$, extrapolated to zero concentration)	10^{7} Diffusion coefficient ($D_{20,w}$ extrapolated to zero concentration)	Average molecular weight Calculated from S and D	Calculated from amino-acid enrichment
HSA	4·32*	5·9*	67,000*	
518,p'TyrHSA	4·35	5·89	66,500	68,950
519,p'TyrHSA	4·40	5·71	69,500	71,730
520,p'TyrHSA	4·55	5·58	73,500	75,960
525,pAlaHSA	4·70	4·90	81,500	83,450

* From Pedersen.[22]

The immunization was started 1 month after the last tolerogenic injection. Each material tested was injected into six rabbits in the test group. After pre-immunization bleedings, the antigen was administered into the thighs of the hind legs of the animals. Three injections of 1·0 ml of the adjuvant mixture were given at fortnightly intervals; 3 weeks after the third injection 20 mg of antigen dissolved in 1 ml of 0·9 per cent sodium chloride was intravenously administered.

The animals were bled 1 day prior to each immunogenic injection and their sera were tested qualitatively. One week after the intravenous injection, sera were collected for quantitative precipitin tests.

Precipitin studies. Qualitative and quantitative precipitin reactions were carried out on sera of individual rabbits. In qualitative tests increasing amounts of antigen (5, 10, 25 and 50 μg) dissolved in 0·5 ml of 0·9 per cent sodium chloride were added to 0·2 ml of antiserum. The contents of the tubes were mixed, placed in a water bath at 37° for 1 hr, and precipitate formation was recorded after 24 hr at 5°. Quantitative precipitin tests were carried out in a final volume of 1·5 ml. Increasing amounts of antigen were added to a constant volume of serum (0·2, 0·5 or 1·0 ml, depending on the immune response), the tubes were mixed, placed in a water bath at 37° C for 1 hr, and then at 5° C for 48 hr. The tubes were centrifuged and the precipitates which formed were washed three times with chilled (2° C) aqueous 0·9 per cent sodium chloride and dissolved in 0·1 N sodium hydroxide (1·1 ml). The extinction (E) of these solutions was read at 2800 Å.

TABLE 3. IMMUNOLOGICAL REACTIONS OF RABBIT ANTI-HSA WITH POLYPEPTIDYL PROTEINS

Anti-HSA of		Test antigen							
		RSA	539,pAlaRSA	HSA	518,p'TyrHSA	519,p'TyrHSA	520,p'TyrHSA	525,pAlaHSA	BSA
Rabbit 66	mg antibody ml serum	0	0	1·86	2·10	1·78	1·82	1·70	
	% cross-reaction				113	95·5	98	91·5	
Rabbit 67	mg antibody ml serum	0	0	5·60	5·70	5·08	4·90	2·65	0·35
	% cross-reaction				102	90·7	89	49	6·3

The amount of antigen in the precipitates was determined in several cases after labelling with ^{131}I. The antibody content was then obtained from the measured E after deducting the calculated E of the antigen.

Farr Technique. Binding to antibodies was estimated also by means of the labelled antigen technique of Farr,[25] as modified by Terres and Wolins.[26] The labelling was carried out with ^{131}I. The radio-iodinated albumins were employed in a concentration of 20 μg/ml.

Radioactivity. Radioactivity of antigen–antibody precipitates, as well as the supernatant fluids, and of the ammonium sulfate precipitates formed in the Farr technique, was measured in a well-type Tracerlab Scintillation Counter.

Sedimentation analysis. This was carried out in a Spinco model E ultracentrifuge, at 20–22°, with a Schlieren optical system. The samples were sedimented at 59,780 rev/min. The results were corrected to 20°.

Diffusion measurements. These were performed in the same Spinco model E ultracentrifuge, according to Daniel and Katchalski.[27] The boundary between the solvent and the solution was obtained with a synthetic-boundary cell and operating at low gear (9341 rev/min). At this speed the sedimentation of the protein derivative is practically negligible.

Amino-acid analysis. The enrichment of a polyalanyl protein with alanyl residues was determined by quantitative analysis[28] with the Beckman-Spinco automatic amino-acid analyser model 120B, Beckman Instruments, Inc., Palo Alto, California. The samples to be analysed were first subjected to hydrolysis with 6 N HCl in sealed tubes at 110° C for 24 hr.

Deamination of polypeptidyl protein. In order to determine the number of amino groups in an albumin which might be unreactive as polyamino-acid side-chain initiators, the polypeptidyl albumins were treated with nitrous acid. The protein (5–6 mg) was dissolved in 0·5 ml of water; 1·5 ml of saturated aqueous $NaNO_2$ and 0·5 ml of glacial acetic acid were added, and the mixture allowed to stand overnight at room temperature. The entire preparation was then dialysed against distilled water and lyophilized, and after hydrolysis in sealed tubes in 6 N HCl at 110° C for 24 hr, it was analysed quantitatively as above.[28]

RESULTS

Immunological reactions of rabbit anti-HSA and anti-polypeptidyl HSA with polypeptidyl proteins and with HSA

The experiments reported in the present study were concerned with the immune responses elicited by polypeptidyl proteins in animals made tolerant to the protein carriers. Since pTyrHSA and pAlaHSA were the main immunogenic materials applied in this investigation, experiments were first performed to test whether the enrichment of HSA with tyrosine or with alanine peptides has not masked the original antigenicity of the native HSA. Rabbit anti-HSA serum was tested for cross-reactivity with 3, 7 and 13 per cent pTyrHSA, and with pAlaHSA, as compared with the precipitins formed with HSA. The results (Table 3) showed that the degree of cross-reactivity with pTyrHSA was similar to the reactivity with HSA. It can, thus, be concluded that the attachment of peptides to HSA did not mask significantly its original specific determinants. This is also inferred from the reciprocal tests: antibodies to polypeptidyl HSA preparations cross-reacted strongly with

HSA (Tables 4, 5; Figs. 1–4). This indicates that not only the antigenic specificity of the native HSA, but also the immunogenic capacity of HSA has been preserved to a great extent when this protein was enriched with polypeptide chains.

TABLE 4. QUALITATIVE PRECIPITIN TESTS IN NORMAL AND TOLERANT RABBITS AT VARIOUS STAGES OF IMMUNIZATION

Group	Immunogen		Test antigen: Homologous adj (1)	adj (2)	adj (3)	i.v.	HSA adj (1)	adj (2)	adj (3)	i.v.
1	HSA	control	4/4*	4/4	4/4	4/4	4/4	4/4	4/4	4/4
		tolerant	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
2	3% p'TyrHSA (518)	control	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
		tolerant	0/6	0/6	0/6	2/6	0/6	0/6	0/6	1/6
3	7% p'TyrHSA (519)	control	2/4	4/4	4/4	4/4	1/4	4/4	4/4	4/4
		tolerant	0/6	2/6	3/6	5/6	0/6	2/6	2/6	4/6
4	13% p'TyrHSA (520)	control	5/6	5/6	6/6	6/6	2/6	5/6	6/6	6/6
		tolerant	0/6	3/6	4/6	6/6	0/6	0/6	1/6	4/6
5	24% pAlaHSA (525)	control	1/6	5/6	6/6	6/6	0/6	3/6	4/6	6/6
		tolerant	0/5	0/4	0/4	2/4	0/5	0/4	0/4	0/4

* Number of rabbits reacting positively per number of rabbits tested.

Antibody production towards polypeptidyl proteins in animals tolerant to the protein carriers

The analysis of the immune responses to polypeptidyl proteins produced in animals tolerant to the protein carriers, was carried out within the following experimental scheme: Rabbits made tolerant to HSA and normal, non-tolerant controls, were divided into 5 experimental groups:

(1) Immunized with HSA, to test the persistence of the tolerant state.
(2) Immunized with the 3 per cent p'TyrHSA (518).
(3) Immunized with 7 per cent p'TyrHSA (519).
(4) Immunized with 13 per cent p'TyrHSA (520).
(5) Immunized with 24 per cent pAlaHSA (525).

The successive appearance of rabbits forming precipitating antibodies following each of the three adjuvant test-immunizations, and the fourth intravenous immunization, are recorded in Table 4. The results indicate that animals made tolerant to HSA retained the tolerant state when further immunized with HSA (group 1). On the other hand, animals which had been made tolerant to HSA, and then challenged with p'TyrHSA, formed antibodies which: (a) reacted with the homologous polypeptidyl conjugate, and (b) reacted with the carrier protein, HSA, i.e. they manifested a breakdown of the previously acquired tolerant state. The immune response elicited by the conjugated proteins seemed correlated (a) with

TABLE 5. PRODUCTION OF ANTIBODIES IN NORMAL AND HSA-TOLERANT RABBITS INJECTED WITH DIFFERENT POLYPEPTIDYL PROTEINS

Group	Injection with	Normal Rabbits					Rabbits tolerant to HSA				
		Rabbit no.	mg antibody* ml serum to			Ratio of anti-HSA to homologous antibody	Rabbit no.	mg antibody* ml serum to			Ratio of Anti-HSA to homologous antibody
			homologous	HSA	523,pTyrRSA			homologous	HSA	523,pTyrRSA	
2	3%pTyrHSA (518)	71	2·55	3·10	0	1·21					
		72	1·96	1·39	0·07	0·71	7	0·83	0·55	0·09	0·66
		73	1·22	1·16	0	0·95	8	0·28	0	0·30	0
3	7%pTyrHSA (519)	63	2·20	2·24	0	1·02	9	0·31	0·20	0·10	0·65
		64	7·40	5·35	0·10	0·86	11	1·02	0·70	0	0·69
		65c	0·70	1·02	0	0·69	12	2·40	1·57	0	0·66
		65z	1·65	1·47	0	0·89	18	0·40	0·10	0	0·25
4	15%pTyrHSA (520)	74	2·85	2·74	0	0·96	23	0·48	0·12	0	0·25
		75	1·02	0·82	0	0·80	33	0·50	0·18	0	0·36
		78	1·85	1·79	0	0·97	36	0·88	0·62	0	0·71
		79	2·52	2·15	0	0·85	41	1·15	0·25	0	0·22
5	24%pAlaHSA (525)	57	1·95	1·00		0·51					
		59	0·53	0·25		0·47	24	0·06	0		0
		60	0·86	0·67		0·78	29	0·13	0		
		61	0·67	0·20		0·30					0

* The amount of antibody was obtained from the optical density at 2800 Å after deducting the calculated optical density of the antigen. The amount of antigen in the precipitate was obtained from radioactivity data (trace-labelling with ^{131}I).

the degree of enrichment of the HSA, with tyrosine residues, and (b) with the extent of immunization. Thus, tolerant animals challenged with the poorly enriched HSA, the 3 per cent p'TyrHSA (518), (Table 4, group 2) retained their tolerance to HSA throughout the first three immunizations; and only after the fourth intravenous challenge, two out of the six rabbits produced antibody to the homologous immunogen; of these only one reacted with HSA. These results show that 3 per cent enrichment with tyrosine conferred a rather weak immunogenicity on the polypeptidyl protein, when tested in animals which had been made tolerant to the protein carrier. Further tyrosination of the HSA significantly increased the immunogenic effect of the polypeptidyl protein, both in terms of numbers of reacting animals, and of time of appearance of antibody as a function of numbers

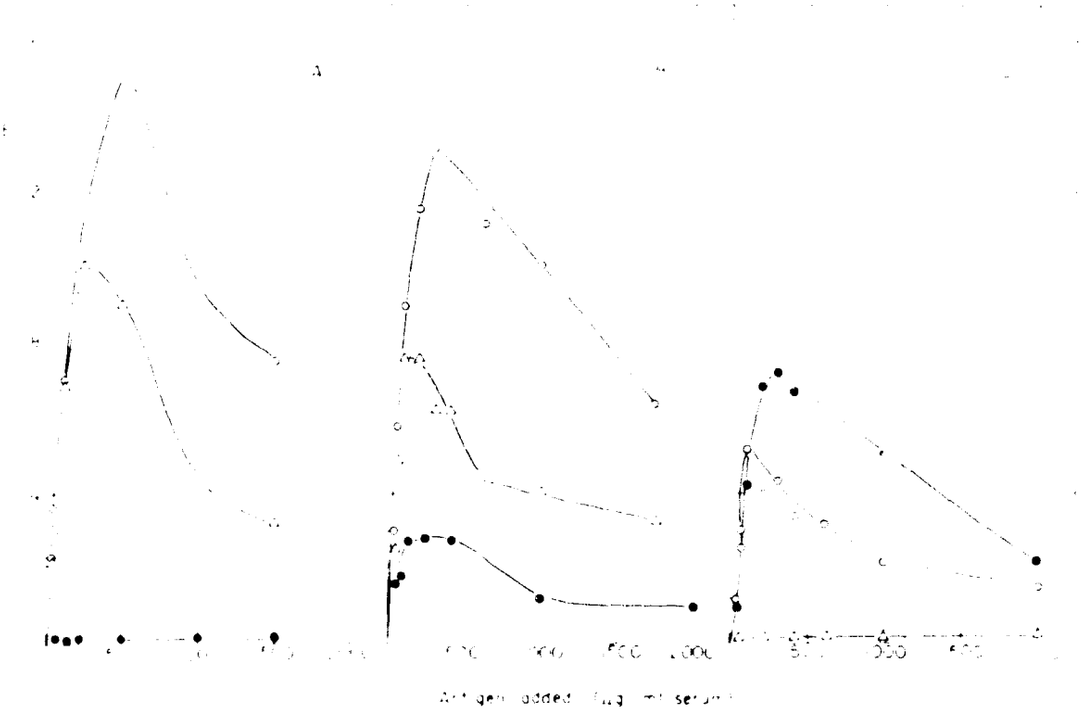

FIG. 1. Extinction at 2800 Å of solutions in 0·1 N sodium hydroxide of precipitates obtained by the addition of — —, 518, 3 per cent p'TyrHSA; — —, HSA; ● —, 23, 14% p'TyrRSA — to antisera against 518 produced by: A, normal rabbit (No. 73); B, rabbit tolerant to HSA (No. 7); C, rabbit tolerant to HSA (No. 8).

of immunizing injections. Immunization with 7 per cent p'TyrHSA (519) (Table 4, group 3) elicited, in five out of six tolerant animals antibody to the homologous antigen. The number of reactive animals increased with successive injections, already beginning after the second challenge; of these four gave precipitin reactions with HSA, again, starting after the second injection. Thus, the immunization with 7 per cent p'TyrHSA (519) was very rapidly associated with the termination of tolerance to HSA. Essentially similar results were obtained when tolerant animals

were challenged with 13 per cent p'TyrHSA (520) (Table 4, group 4). Here, however, the formation of antibody to the polypeptidyl determinant as a function of the number of antigen injections, preceded the formation of antibody to HSA. The termination of tolerance, i.e. the formation of antibodies reacting with HSA *followed* the production of antibody to the polytyrosyl determinant.

The formation of antibody to the polypeptidyl components in tolerant animals, and the termination of tolerance, seemed to be determined by the chemical nature of the amino-acid residues attached. Thus, when rabbits tolerant to HSA were immunized with 24 per cent pAlaHSA (525) (Table 4, group 5)—only two out of

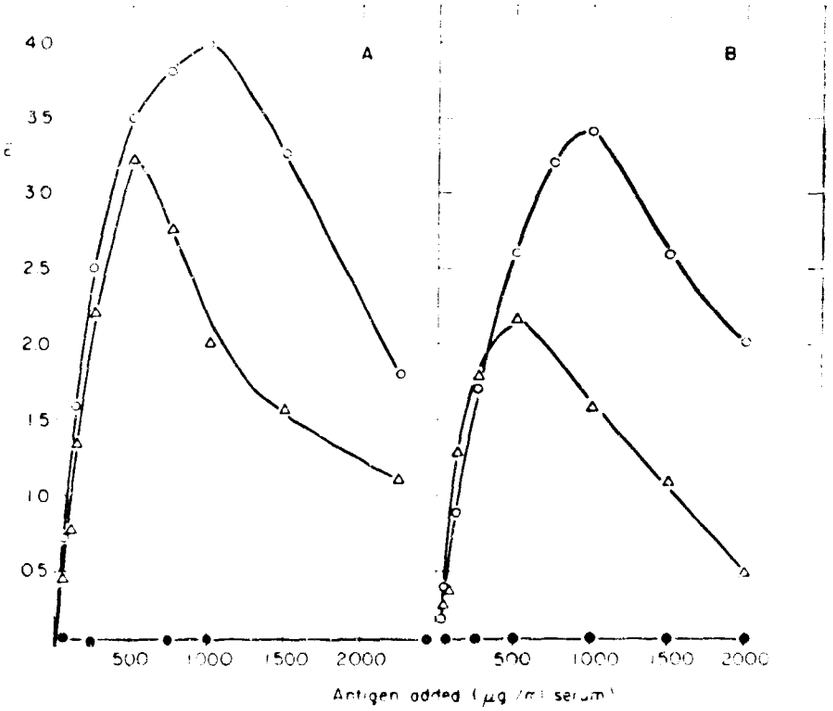

Fig. 2. Extinction at 2800 Å of solutions in 0·1 N sodium hydroxide of precipitates obtained by the addition of— , 519, 7 per cent p'TyrHSA; , HSA; ●, 523, 14 per cent p'TyrRSA—to antisera against 519 produced by: A, normal rabbit (No. 63); B, rabbit tolerant to HSA (No. 12).

four produced some antibody to the homologous antigen, and none formed antibody to HSA (Table 5, group 5). The pAlaHSA, under the experimental conditions tested, did not break up tolerance to HSA. It is pertinent that in normal animals, immunization with the sample of pAlaHSA resulted in a much higher production of antibodies and that these antibodies cross-reacted well with HSA.

It should be noted, that all sera which were negative against HSA in the precipitin tests, were also negative when tested by means of the Farr method, namely by testing the binding capacity of the antisera to ^{131}I-labelled HSA.

An analysis of the correlation between the degree of enrichment with tyrosine residues and the termination of tolerance to HSA, was carried out by means of the quantitative precipitin tests (Figs. 1, 2, 3 and Table 5).

Figure 1 presents the precipitin curves of the two tolerant rabbits, which produced antibodies to the homologous conjugate, following immunization with 3 per cent p'TyrHSA (518). The ratio of anti-HSA to anti-p'TyrHSA in the only tolerant animal in which anti-HSA has been produced, is similar to that found in the non-tolerant control animals. This similarity was also demonstrated when tolerant rabbits were immunized with the 7 per cent p'TyrHSA (519) (Fig. 2 and Table 5, group 3). When, however, tolerant rabbits were immunized with HSA which was more highly enriched (13 per cent) with tyrosine residues, the formation of anti-HSA was decreased (Fig. 3 and Table 5, group 4).

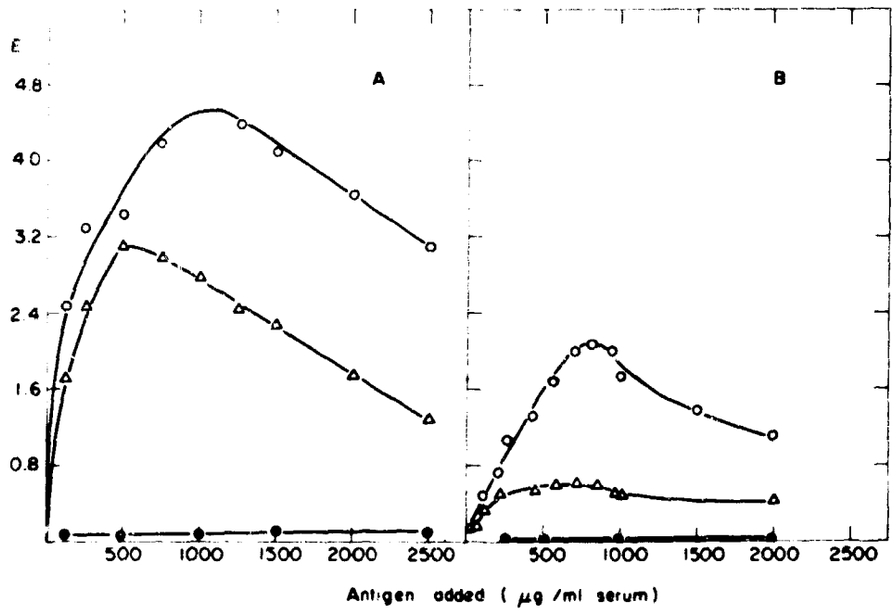

FIG. 3. Extinction at 2800 Å of solutions in 0·1 N sodium hydroxide of precipitates obtained by the addition of— , 520, 13 per cent pTyrHSA; , HSA; ●, 523, 14 per cent pTyrRSA—to antisera against 520 produced by: A, normal rabbit (No. 79); B, rabbit tolerant to HSA (No. 41).

The final incidence of HSA-tolerant animals producing anti-HSA, following immunization with 13 per cent p'TyrHSA (520), was found similar to the incidence of tolerant rabbits producing anti-HSA following immunization with 7 per cent p'TyrHSA (519) (Table 4). Yet the kinetics of tolerance breakdown as a function of the number of antigen injections was different (Table 4): those immunized with 7 per cent p'TyrHSA showed an earlier termination of tolerance to HSA than those challenged with 13 per cent p'TyrHSA. Furthermore, the amount of antibody to HSA produced by the individual rabbits was significantly lower in animals immunized with 13 per cent p'TyrHSA, as compared to those immunized with 7 per cent p'TyrHSA (Table 5, groups 3, 4). Thus, the degree of tolerance breakdown was, within certain levels of enrichment with tyrosine (from 3 to 7 per cent)—proportionally correlated with the degree of enrichment with the tyrosine residues. Further enrichment with tyrosine (from 7 to 13 per cent) decreased the reactivation

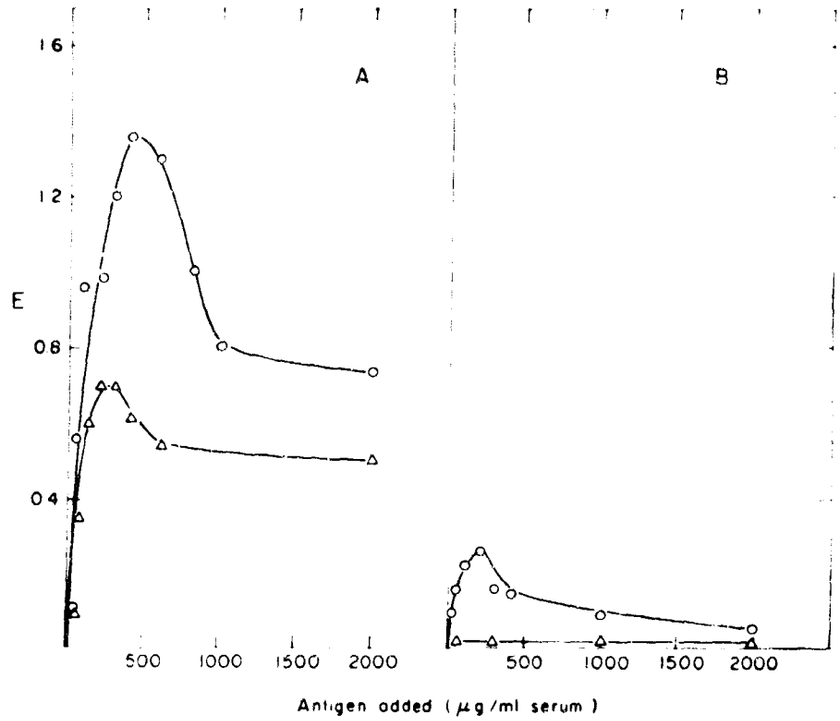

Fig. 4. Extinction at 2800 Å of solutions in 0·1 N sodium hydroxide of precipitates obtained by the addition of— , 525, 24 per cent pAlaHSA; ---, HSA—to antisera against 525 produced by: A, normal rabbit (No. 60); B, rabbit tolerant to HSA (No. 29).

Table 6. Antibodies to poly-DL-alanyl determinants demonstrated in cross-reaction tests

Test antigen	anti-pAlaHSA (525)		anti-pAlaRSA (539)
	tolerant	control	
pAlaHSA (525)	3·4* (6·1)†	5·5 (19)	3·3 (7·1)
HSA	0·4	4·4 (2·2)	0·3
pAlaRSA (539)	2·4 (5·5)	5·5 (12)	3·3 (6·5)
pAlaRNase	2·4 (2·9)	5·5 (7)	3·3 (4·5)
RNase	0·4	0·5	0·3

* Number of rabbits reacting positively per number of rabbits tested.

† Microgram antigen bound per ml serum (Farr technique), average value.

of the immune response to HSA. The formation of antibodies in rabbits tolerant to HSA and immunized with 24 per cent pAlaHSA (525) is presented in Fig. 4 and Tables 4 and 5. Although no anti-HSA antibodies have been produced in tolerant animals, antibodies to the poly-DL-alanyl determinant could be demonstrated. These findings were verified with the aid of the Farr technique (Table 6).

Response to polypeptidyl determinants in animals tolerant naturally or experimentally to the protein carriers

The immunological response induced by polypeptidyl determinants, when attached to a protein towards which the rabbits are naturally tolerant (i.e. RSA), was compared to the immunological response produced by the same polypeptidyl determinant, when attached to a protein towards which the animals had acquired tolerance experimentally (i.e. HSA).

Anti-pTyrHSA produced in animals tolerant to HSA and anti-pTyrRSA, were tested for cross-reactivity with pTyrGel and pTyrRSA. Since rabbit anti-HSA does not cross-react with gelatin or with RSA, antibodies demonstrated in such tests should be attributed to the polytyrosyl determinants alone. The results of such tests, carried out by the Farr technique, are presented in Table 7. Anti-pTyrHSA, produced by the 3, 7 and 13 per cent-enriched HSA, bound the ^{131}I-labelled pTyrGel at levels similar, in order of magnitude, to the binding of the same antigens by antisera produced by pTyrRSA. These antisera manifested the same order of binding capacity also when tested with pTyrRSA. Thus, animals which are naturally tolerant to RSA, as well as animals which had acquired tolerance to HSA, produced antibodies to the tyrosine peptide determinants, when immunized with the respective tolerogenic proteins enriched with tyrosine residues. In this respect, therefore, there was no difference in the manifestations of the recognition mechanism, between natural and acquired tolerance.

A similar experimental approach was applied in testing anti-pAlaHSA produced in rabbits tolerant to HSA and anti-pAlaHSA obtained in normal rabbits. The binding capacities of such antisera to ^{131}I-labelled pAlaHSA, pAlaRSA and pAlaRNase are presented in Table 6. Anti-pAlaHSA produced in HSA-tolerant rabbits and antpAlaRSA did not show any binding capacity when tested with HSA or RNase (and, obviously, these antisera could not bind RSA). Yet, the two antisera did bind the labelled pAlaHSA, pAlaRSA and pAlaRNase. This indicates that anti-polyalanine has been produced in tolerant animals, when immunized with antigens in which alanine peptides are attached either to a protein towards which the rabbits are naturally tolerant, or to proteins towards which the rabbits had acquired tolerance. Here, again, the immunological manifestations of natural and acquired tolerance are basically the same.

DISCUSSION

The experiments reported in the present study have demonstrated that acquired tolerance to HSA could be terminated if the unresponsive rabbits are immunized with polyamino-acid conjugates of HSA. Two parameters appear to determine the breakdown of tolerance: (1) the chemical nature of the attached polyamino acid: pTyrHSA was effective in the termination of tolerance, whereas pAlaHSA, under the experimental conditions tested, was not; (2) the degree of enrichment with the

TABLE 7. ANTIBODIES TO POLY-L-TYROSYL DETERMINANTS DEMONSTRATED IN CROSS-REACTION TESTS

Test antigen	anti-14% p'TyrRSA (523) tolerant	anti-14% p'TyrRSA (523) control	anti-3% p'TyrHSA (518) tolerant	anti-3% p'TyrHSA (518) control	anti-7% p'TyrHSA (519) tolerant	anti-7% p'TyrHSA (519) control	anti-13% p'TyrHSA (520) tolerant	anti-13% p'TyrHSA (520) control
15% p'TyrGel (514)	4/5* (7·5)†	5/6 (9)	1/6 (14·3)	2/4 (10·9)	5/6 (13·6)	3/4 (14·2)	2/5 (5·8)	
14% p'TyrRSA (523)	5/5 (15·2)	6/6 (18·6)	2/6 (11·4)	1/4 (9·5)	3/6 (6·6)	4/4 (5)	5/5 (5·2)	5/5 (27·0)

* Number of rabbits reacting positively per number of rabbits tested.
† Micrograms antigen bound per ml serum (Farr technique), average value.

appropriate residues. The capacity of pTyrHSA to terminate tolerance to HSA is correlated with the capacity of tyrosine peptides to confer antigenicity on antigenically weak proteins,[11,29] while the incapacity of alanine peptides to help the breakdown of tolerance might be correlated with their lack of capacity to convert gelatin into a potent immunogen.[11] Thus, pTyrHSA broke tolerance to HSA, in contrast to pAlaHSA which was unable to terminate tolerance under the experimental conditions of the present study.

The degree of enrichment with tyrosine was shown to determine the level of tolerance breakdown. Enrichment with 3 per cent of tyrosine residues caused formation of anti-HSA in only one out of six animals, whereas the 7 per cent enrichment with tyrosine terminated tolerance in four out of six rabbits, starting already after the second immunization. Further enrichment (13 per cent), however, decreased somewhat the capacity of the conjugate to elicit antibodies to HSA in HSA-tolerant animals. Here, the formation of anti-polytyrosyl antibodies preceded the production of anti-HSA; and when anti-HSA antibodies had been detected they were of a lower level than those produced by the 7 per cent pTyrHSA. It is, therefore, concluded that there is an optimal degree of molecular alteration which will confer on the altered antigen the maximal potency to terminate tolerance.

The level of tolerance breakdown, obtained by pTyrHSA, was significantly greater than the level of breakdown obtained previously by other chemically altered proteins, such as azo-conjugates. Weigle[7] achieved the most potent reactivation of anti-BSA, in BSA-tolerant animals, following immunization with an arsanil-sulfanil-BSA. However, the ratio of anti-BSA to the antibody to the homologous antigen was in 7/9 tolerant animals significantly lower than in the normal controls. On the other hand, in our system, when tolerance was terminated by pTyrHSA enriched with 3 or 7 per cent tyrosine residues, the ratio of anti-HSA to antibodies to the homologous antigens was in 4/5 animals of the same order in the tolerant and in the control animals. It appears, therefore, that the level of tolerance breakdown as measured by this ratio was definitely greater than that reported for azo-conjugates of serum proteins or, in fact, by any other system which was shown to terminate tolerance.[6,8,30]

The ratio of antibody production to HSA and to pTyrHSA in the tolerant and control animals should be considered in relation to the possible cellular basis of immunological tolerance. One of the central problems of immunological tolerance is whether there is only a tolerant organism, from which certain predetermined cells have been eliminated,[31,32] or whether there are tolerant cells *per se*.[33,34] According to the clonal selection concept, the tolerant state is based on the elimination of specific predetermined clones. The immunological reactivation would then depend on the reappearance of clonal stem cells which would have to replicate, i.e. to form 'clones' of proper cell populations, prior to the formation of detectable amount of antibody. If cells predetermined to form anti-HSA have been eliminated from the organism in HSA-tolerant animals, whereas those capable of forming anti-polytyrosine have not, then the reactivation will depend on the gradual regeneration of clones capable of forming anti-HSA. The sizes of the cell populations capable of forming anti-HSA in the tolerant animals should be smaller during the 'regeneration' process than those capable of forming anti-HSA in the normal animals.

Accordingly, it would be expected that the ratio of anti-HSA to anti-pTyrHSA in the normal animals, would be different from that ratio in the tolerant animals. Yet, we found that when a proper breakdown has been achieved, the ratio of the two groups was of the same order. If this similarity represents a similarity in the sizes of cell populations engaged in antibody production, then our results are not compatible with the notion that tolerance is based on the elimination of cells. They are compatible with the notion that tolerant, specifically non-reactive cells, *do exist*, and the experimental breakdown is based on the reactivation of these cells which previously were non-reactive to HSA.[35]

The experimental termination of acquired tolerance may be relevant to the termination of natural tolerance, if the two systems are based on a similar mechanism. So far, however, very little is known on the mechanism of immunological tolerance—and the similarity between acquired and natural tolerance can be deduced only from the similarity in the immunological manifestations following various experimental treatments. Indeed, applying this approach, Boyden and Sorkin[5] claimed to have obtained different responses to the haptenic determinants when testing azo-conjugates of proteins towards which the animals were made tolerant, as compared to those towards which the animals are naturally tolerant. Nachtigal and Feldman,[6] on the other hand, found a complete similarity between these two systems, when analysing the responses to the haptenic groups. In the present study further evidence is brought, indicating that the immunological manifestations of the two systems are similar: antibodies to the polypeptidyl determinants (both Tyr and Ala) were obtained with RSA-conjugates, as well as with HSA-conjugates injected to HSA-tolerant rabbits. If, in fact, this similarity represents a basic similarity in the mechanism of the two systems of tolerance, then the results obtained in the experimental termination of acquired unresponsiveness may be relevant to the termination of natural tolerance.

SUMMARY

A study was made of the immune responses to polypeptidyl proteins in rabbits which were either naturally tolerant or made experimentally unresponsive to the protein carriers. Experimentally acquired tolerance to HSA could be terminated by immunization of the unresponsive rabbits with poly-L-tyrosyl derivatives of HSA. Two parameters determined the breakdown of tolerance: (1) the chemical nature of the peptides attached: polytyrosyl HSA was effective in the termination of tolerance to HSA, whereas polyalanyl HSA was not; (2) the degree of enrichment with tyrosine. There appears to be an optimal degree of enrichment, i.e. of molecular alteration of the HSA, which will confer on the altered antigen the maximal potency to terminate tolerance: a slight alteration (3 per cent enrichment of HSA with Tyr) and a severe alteration (13 per cent enrichment) were less effective in tolerance breakdown than an intermediate degree of alteration (7 per cent enrichment with Tyr). The level of tolerance breakdown obtained by polytyrosyl HSA, as measured by the ratio of anti-HSA anti-polytyrosyl HSA, was greater than the level obtained previously by other chemically altered antigens. The significance of this ratio was discussed in relation to the cellular basis of immunological tolerance. Antibodies were produced to the peptides *per se*, when attached to proteins towards which the animal is naturally tolerant (RSA), or to proteins to which the animal has acquired

tolerance (HSA). In both systems, there was a similar pattern of antibody formation, which may reflect a similarity in mechanism between natural and actively acquired tolerance.

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